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Introduction

Breast cancers are very poor in initiating effective immune responses despite expressing antigens recognizable by a host's immune system. We have recently identified novel members of the B7 gene family, PD-Ligand 1 and PD-Ligand 2, that are ligands for PD-1 and can inhibit T cell proliferation and cytokine production (1, 2). PD-1 has structural similarity to CTLA-4, and has an immunoreceptor tyrosine based inhibitory motif (ITIM) in its cytoplasmic tail. PD-1 is expressed on activated T cells, B cells and myeloid cells. The phenotype of PD-1^{-/-} mice demonstrates the inhibitory role of PD-1 *in vivo*. PD-L1 is overexpressed on breast cancer cells.

Our hypothesis is that PD-L1 expression on breast cancer cells engages PD-1 on activated T cells and leads to inhibition of the anti-tumor immune response. We hypothesize that T cell activation is determined by a summation of positive signals provided by the strength of the TCR signal and costimulatory signals, primarily provided by CD28, and inhibitory signals mediated by molecules such as CTLA-4 and PD-1. The problem with generating immune responses to tumor antigens is that most are self antigens and high affinity TCR have been eliminated by thymic selection. Nevertheless, low affinity anti-self TCR are present in the peripheral T cell population. We hypothesize that blockade of immunoinhibitors like PD-Ligand will lower the threshold of T cell activation such that responses to weak tumor antigens are generated. In support of this idea, blockade of CTLA4, another immunoinhibitory receptor structurally similar to PD-1, has been shown to lead to the generation of anti-tumor immune responses and the rejection of tumors. Our objective is to test whether breast cancer cells use PD-Ligands to inhibit the anti-tumor response and to block this inhibitory signal in order to stimulate an anti-tumor response.

Body

1. Expression of PD-Ligands on breast cancer cell lines and breast cancer patients: We have shown the expression of PD-L1 on Breast cancer cell lines MDA231, SKBR-3, MCF-7 but not on BT474. We developed anti-PD-L2 antibody. PD-L2 expression on breast cancer cell lines was further evaluated together with anti-PD-L1 expression (Fig1). PD-L1 is highly expressed on MDA231, moderately on ZR75.1 while PD-L2 is highly expressed on SKBR3. PD-L1 was expressed on a broad spectrum of carcinomas, tumors of epithelial origin. Nine out of 12 breast cancer patients, including invasive ductal and lobular carcinomas, showed the up-regulated expression of PD-L1. In sections of breast that contained both normal (thin arrow, Figure 2) and malignant cells (thick arrow, Figure 2), expression of PD-L1 was much higher on the malignant cells (published in Brown, Dorfman, Ma, et al, J. Immunol.)

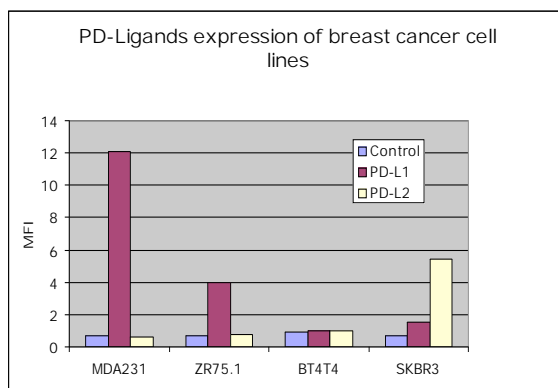


Fig. 1

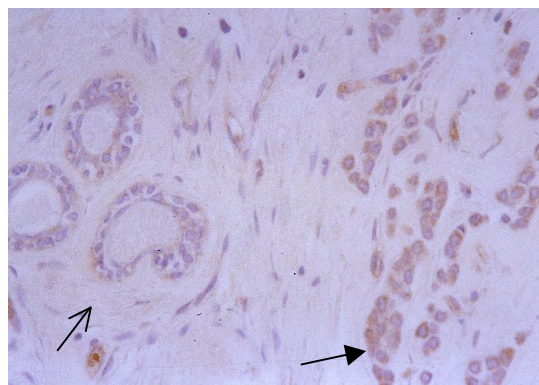


Fig. 2

2. Regulation of PD-L1 expression on MDA231 cell lines by estrogen. Studies have shown that prolonged exposure to cycling estrogen and progesterone levels contributes to the development of breast cancer. Removal of endogenous estrogen via oophorectomy decreases the risk of breast carcinoma. We examined whether estrogen could regulate PD-Ligand expression. As shown in Figure 3, at low to moderate levels of estrogen concentration (0-50 μ g/ml), estrogen up-regulates PD-L1 expression. High concentrations of estrogen down-regulate PD-L1 expression. There was little effect on PD-L2 expression. Epitope specificity screening of PD-Ligand antibodies to identify non-overlapping recognition for use in an ELISA: In order to develop an ELISA for soluble PD-Ligand detection (Aim 3 in our proposal), we screened for non-overlapping PD-Ligand antibodies by Ab blocking of surface staining. . In order to accomplish these epitope blocking studies, we made 300.19 and CHO cells transfected with PD-L1 or PD-L2. For anti-PD-L1 monoclonal antibodies, we found 29E.12B1 is a non blocking mAb to 29E.2A3. For anti-PD-L2 monoclonal antibodies, 24F.7G12 is a non blocking antibody to 24F.2D7, 24F.2H7, and 24F.7C8

3. ELISA for serum PD-L1 detection: Based on the two non-overlapping mAbs 29E.12B1 and 29E.2A3, we developed a PD-L1 ELISA. 12B1 was coated at 5µg/ml on 96-well plate. PD-L1-Ig fusion protein was used as standard PD-L1 protein. Biotinylated-2A3 was used as detection Ab, followed by Streptavidin binding. The sensitivity of PD-L1 ELISA is around 4 ng/ml (Fig 4). We did ELISA with sera from four breast cancer patients together with two healthy controls. No significant difference was found in the level of soluble PD-L1 between normals and breast cancer patients.

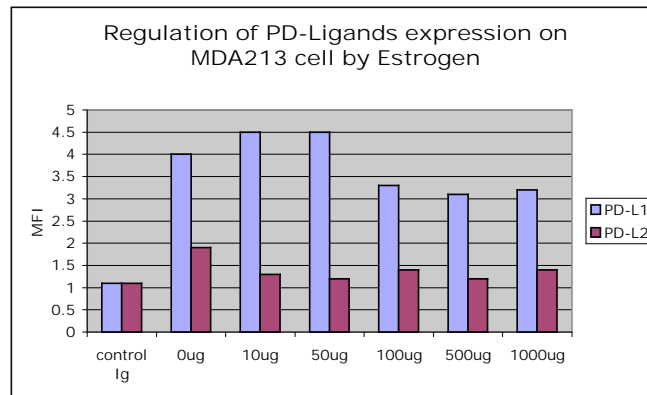


Fig. 3

4. Blockade of the PD-L:PD-1 pathway enhanced CD4⁺ T cell activation in allo-responses. We compared the effect of PD-L blockade on CD4⁺ T cell alloresponses using DC of weak (IL-10 pretreated mDC or iDC) vs strong (mDC) stimulatory capacity. Our data showed that the addition of anti-PD-L mAbs enhanced CD4⁺ T cell proliferation compared with isotype control Abs. Anti-PD-L2 was more effective at increasing T cell proliferation than anti-PD-L1, while the combination of both anti-PD-L Abs provided an additive stimulation of CD4⁺ T cell proliferation (See the supplement article, Brown et al. Figure 6)

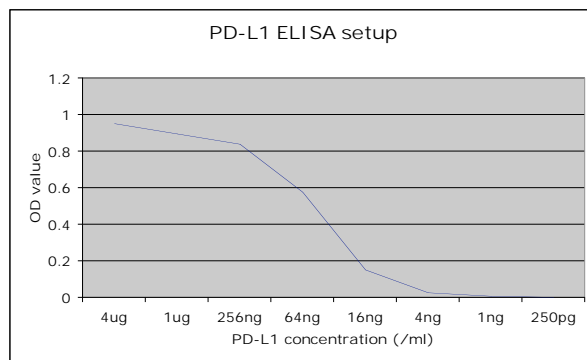


Fig. 4

5. PD-Ligand blockade enhanced CD8⁺ T cell proliferation in an allo-response with MDA231 breast cancer cell line: CD8⁺ T cell responses are particularly important in anti-tumor immunity. Carter et al. have shown that PD-Ligands inhibit CD8 T cell activation more effectively than CD4 T cell activation (3). CD8⁺ T cells were separated from healthy donor leukopack using RosetteSep kit. MDA231 is a human mammary gland adenocarcinoma cell line that we have found highly expresses PD-L1, and also expresses MHC class I but does not express MHC class II. MLR (mixture lymphocyte reaction) was carried out using donor CD8 T cells with mitomycin C inactivated MDA231 cells. MAbs to PD-Ligands or isotype controls were added at 20 µg/ml to the MLR culture. Four days later, T cell proliferation was assayed by ³H incorporation assay (Fig 5). Our data showed that the addition of anti-PD-Ligand Abs up-regulated CD8 T cell proliferation compare with isotype control antibodies.

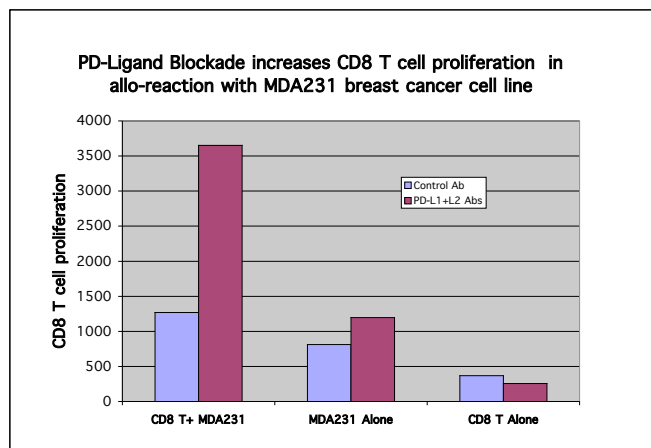


Fig. 5

6. Blockade of the PD-L:PD-1 pathway enhances IFN-γ production by CD8 T cells in the MDA231 MLR assay. Supernatants were collected in the MLR described above and IFN-γ was assayed by ELISA (Fig 6). Consistent with the increase in T cell proliferation, IFN-γ production was also increased with blocking Abs to PD-Ligands.

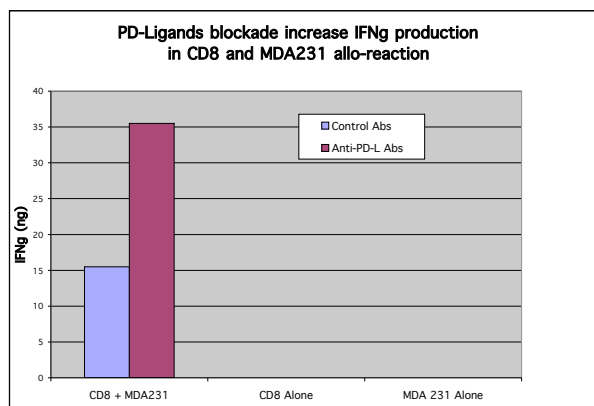


Fig. 6

7. PD-ligand blockade increases CD8 T cell clonal expansion in response to MDA231 cell. CD8 T cells were cultured with Mitomycin C-treated MDA231 cells for 7 days. Proliferating CD8 T cells were re-stimulated with MDA231 cells for another 7 days. At day 14, T cell number was counted (Fig 7). Our data showed that PD-Ligand blockade enhanced T cell clonal expansion, with anti-PD-L2 blockade being more potent than PD-L1 blockade in stimulating T cell proliferation.

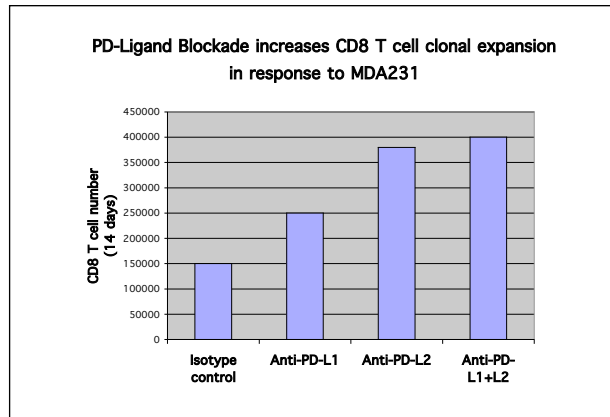


Fig. 7

Key Research Accomplishments

1. PD-L1 but not PD-L2 is highly expressed on the cell surface of breast cancer cells but not on normal breast epithelium. PD-L1 is also highly expressed on most carcinomas.
2. Estrogen modestly increases PD-L1 expression on the MDA-231 breast cancer cell line. PD-L2 expression is unaffected.
3. We epitope mapped anti-PD-L1 and PD-L2 mabs and identified non-overlapping pairs for use in an ELISA for the detection of soluble PD-L1.
4. We developed an ELISA to detect soluble PD-L1 in serum. In the small number of samples analyzed, there was no difference in soluble serum levels of PD-L1 between normals and breast cancer samples.
5. Blockade of the PD-L:PD-1 pathway enhanced CD4⁺ T cell activation in allo-responses.
6. PD-Ligand blockade enhanced CD8⁺ T cell proliferation and cell number in allo-reaction with the MDA231 breast cancer cell line.
7. Blockade of PD-L:PD-1 pathway enhances IFN- γ production in CD8 T cells in allo-reaction with the MDA231 breast cancer cell line.

Reportable Outcomes

Publication:

Julia Brown, David Dorfman, Feng-Rong Ma, Gordon Freeman. Blockade of Programmed Death-1 ligands on Dendritic Cells Enhances T Cell Activation and Cytokine Production. *The Journal of Immunology*, 2003, 170:1257-1266

Conclusions

The PD-1/PD-1 Ligand pathway inhibits T cell activation. We demonstrated expression of PD-L1 by immunohistochemistry on breast tumor cells in situ but PD-L1 was not expressed on normal breast epithelium. PD-L1 expression provides a mechanism whereby tumor cells can inhibit immune responses. We also demonstrated expression of PD-L1 in different breast cancer cell lines. We found MDA231 has the highest PD-L1 expression among the cell lines we tested. SKBR3, another breast cancer cell line, has the most PD-L2 expression. PD-L1 expression on MDA231 cells can be up-regulated by estrogen up to 50 $\mu\text{g/ml}$ but was down-regulated at higher concentrations. We did not find a significant change of PD-L2 expression under estrogen treatment. We identified anti-PD-ligand antibodies with non-overlapping epitopes and successfully developed a sensitive ELISA for detecting soluble PD-L1 in serum. In the small number of samples analyzed, there was no difference in soluble serum levels of PD-L1 between normal controls and breast cancer samples. Blockade of the PD-1/PD-1 Ligand pathway upregulated CD4⁺ and CD8⁺ allo-responses, in which either dendritic cells or MDA231 breast cancer cells were used as allo-stimulators. Blockade of the PD-1/PD-1 Ligand pathway also enhanced CD8 T cell IFN- γ production and an increase in CD8 T cell number in an allo-response to MDA231 cells.

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Appendices

Article:

Julia Brown, David Dorfman, Feng-Rong Ma, Gordon Freeman. Blockade of Programmed Death-1 ligands on Dendritic Cells Enhances T Cell Activation and Cytokine Production. *The Journal of Immunology*, 2003, 170:1257-1266

Blockade of Programmed Death-1 Ligands on Dendritic Cells Enhances T Cell Activation and Cytokine Production¹

Julia A. Brown,* David M. Dorfman,[†] Feng-Rong Ma,* Elizabeth L. Sullivan,* Oliver Munoz,* Clive R. Wood,[‡] Edward A. Greenfield,* and Gordon J. Freeman^{2*}

Programmed death-1 ligand (PD-L)1 and PD-L2 are ligands for programmed death-1 (PD-1), a member of the CD28/CTLA4 family expressed on activated lymphoid cells. PD-1 contains an immunoreceptor tyrosine-based inhibitory motif and mice deficient in PD-1 develop autoimmune disorders suggesting a defect in peripheral tolerance. Human PD-L1 and PD-L2 are expressed on immature dendritic cells (iDC) and mature dendritic cells (mDC), IFN- γ -treated monocytes, and follicular dendritic cells. Using mAbs, we show that blockade of PD-L2 on dendritic cells results in enhanced T cell proliferation and cytokine production, including that of IFN- γ and IL-10, while blockade of PD-L1 results in similar, more modest, effects. Blockade of both PD-L1 and PD-L2 showed an additive effect. Both whole mAb and Fab enhanced T cell activation, showing that PD-L1 and PD-L2 function to inhibit T cell activation. Enhancement of T cell activation was most pronounced with weak APC, such as iDCs and IL-10-pretreated mDCs, and less pronounced with strong APC such as mDCs. These data are consistent with the hypothesis that iDC have a balance of stimulatory vs inhibitory molecules that favors inhibition, and indicate that PD-L1 and PD-L2 contribute to the poor stimulatory capacity of iDC. PD-L1 expression differs from PD-L2 in that PD-L1 is expressed on activated T cells, placental trophoblasts, myocardial endothelium, and cortical thymic epithelial cells. In contrast, PD-L2 is expressed on placental endothelium and medullary thymic epithelial cells. PD-L1 is also highly expressed on most carcinomas but minimally expressed on adjacent normal tissue suggesting a role in attenuating antitumor immune responses. *The Journal of Immunology*, 2003, 170: 1257–1266.

Mature dendritic cells (mDCs³) express high levels of MHC and costimulatory molecules and are powerful APC that initiate T cell immune responses (1). Surprisingly, recent data have revealed that Ag presentation by immature dendritic cells (iDCs) has a role in establishing and maintaining T cell tolerance (2–5). Despite expressing moderate levels of MHC and costimulatory molecules, presentation by iDCs stimulates the development of regulatory T cell populations (2, 3). Furthermore, IL-10-pretreated mDCs express lower but detectable amounts of MHC and costimulatory molecules and induce T cell anergy in vitro (3, 6, 7). Stimulatory and inhibitory signals presented by dendritic cells (DCs) during Ag presentation are integrated by the T cell and determine the final outcome of T cell activation.

The ability of three members of the B7 gene family, B7-1, B7-2, and inducible costimulator ligand, to provide costimulatory signals during the process of T cell activation has been well characterized (8, 9). Two additional B7 family members, programmed death-1 ligand (PD-L)1 (B7-H1) and PD-L2 (B7-DC), have recently been shown to down-regulate T cell activation through their mutual receptor, programmed death-1 (PD-1) (10–12). PD-1, a CD28 homologue, contains two immunoreceptor tyrosine-based motifs that are phosphorylated upon receptor engagement and recruit Src homology 2-domain-containing tyrosine phosphatase 2 (11, 13–15). Although initially isolated from two cell lines undergoing programmed cell death (16), neither cross-linking of PD-1 by mAb or PD-L results in programmed cell death. Rather, engagement of PD-1 results in cell cycle arrest (11, 12, 17).

Unlike other CD28 family members, PD-1 demonstrates a broad expression pattern and is found on activated T, B, and myeloid cells (18) and on a subset of thymocytes (19). PD-1^{-/-} mice display a variety of autoimmune pathologies, demonstrating the role of PD-1 as a negative regulator of the immune response (15, 20). Engagement of PD-1 by PD-L1-Ig or PD-L2-Ig fusion protein has an inhibitory effect on proliferation and cytokine production during anti-CD3-mediated stimulation (10, 11). Chinese hamster ovary (CHO) cell transfectants capable of presenting OVA peptide in the context of I-A^d and expressing either PD-L1 or PD-L2 with or without B7-2 costimulation inhibit the proliferation and cytokine production of DO11 TCR transgenic T cells, particularly at low concentrations of Ag (11). The PD-L:PD-1 pathway inhibits proliferation by reducing the production of IL-2 and restricts the number of T cells that gain entry into the cell cycle as well as their subsequent division rate (12). In contrast to these results, Dong et al. (21) have reported that PD-L1-Ig (B7-H1-Ig) will moderately stimulate T cell proliferation and strongly up-regulate IL-10 production. Tseng et al. (22) have reported that PD-L2-Ig (B7-DC-Ig)

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³ Abbreviations used in this paper: mDC, mature dendritic cell; iDC, immature dendritic cell; DC, dendritic cell; FDC, follicular DC; PD-1, programmed death-1; PD-L, PD-1 ligand; CHO, Chinese hamster ovary; DN, double negative; DP, double positive.

strongly up-regulates T cell activation and IFN- γ production. Additional experiments with blocking mAbs and knockout mice are needed to resolve these differences.

In this study, we report the development and characterization of mAbs that specifically recognize either human PD-L1 or PD-L2. We demonstrate that blockade of PD-L1 and PD-L2 on various populations of monocyte-derived DCs enhances CD4⁺ T cell proliferation and cytokine production. Both whole mAb and Fab enhance T cell activation. Additionally, we show that PD-L are expressed much more broadly in normal tissues than are other members of the B7 gene family. A variety of epithelial-derived tumors also express PD-L1, suggesting that these malignancies may exploit the PD-L:PD-1 pathway to attenuate antitumor immunity.

Materials and Methods

Generation of anti-human PD-L1 and PD-L2 mAbs and Fab fragmentation

mAbs to human PD-L1 have been described previously (11). mAbs to human PD-L2 were produced similarly. Female BALB/c mice were prepared for cDNA immunization by injecting 50 μ l of 10 mM cardiotoxin (Sigma-Aldrich, St. Louis, MO) in 0.9% saline into the tibialis anterior muscle of each hind limb. Five days later, 50 μ l of 1 mg/ml purified human PD-L2 cDNA in the pAXEF mammalian expression vector in 0.9% saline was injected into the regenerating tibialis anterior muscle of each mouse. The cardiotoxin and cDNA immunization was repeated six times at 2- to 4-wk intervals. Spleen cells were fused with SP2/0 myeloma cells and cloned, and hybridoma supernatants were screened by cell surface staining of PD-L2 transfected 300.19 and CHO cells and for lack of reactivity with vector-alone transfected cells. Clones 29E.2A3 (anti-PD-L1; IgG2b), 29E.5A9 (anti-PD-L1; IgG1), and 24F.10C12 (anti-PD-L2; IgG2a) were chosen as reagents for FACS analysis and functional studies. PD-L transfected cells were made as previously described (11). Fab fragmentation was performed using immobilized papain (Pierce, Rockford, IL), and purification of the Fab was achieved by removal of Fc with protein A. The purity of the Fab was determined by HPLC to be >95%. Anti-PD-L1 and anti-PD-L2 whole mAb and Fab were subjected to reducing SDS-PAGE (5 μ g/lane), and the gel was stained with Coomassie blue. Fig. 2 demonstrates that no contaminating whole mAb was present in the Fab preparations. Fab retained their capacity to bind to PD-L. FACS analysis with whole anti-PD-L1 and anti-PD-L2 mAb and their Fab revealed approximately the same mean fluorescence intensity upon binding to PD-L1 or PD-L2 transfected cells, respectively (Figs. 1 and 2).

Blockade of PD-1-Ig binding by anti-PD-L1 or anti-PD-L2 mAb

To determine if anti-PD-L1 or anti-PD-L2 mAbs could block the interaction between PD-1-Ig and PD-L, a competitive binding experiment was performed. 300.19 transfectants that express PD-L1 or PD-L2 were preincubated with anti-PD-L1 or anti-PD-L2, respectively, followed by the addition of biotinylated PD-1-Ig. Cells were washed twice, incubated with streptavidin-PE, washed again, fixed with 2% paraformaldehyde, and analyzed on a Coulter Epics XL (Beckman Coulter, Miami, FL). Cells incubated with biotinylated PD-1-Ig and mouse IgG, or biotinylated mouse IgG with no biotinylated PD-1-Ig were used as positive and negative controls, respectively. Fig. 1B shows that anti-PD-L1 and anti-PD-L2 block the binding of PD-1-Ig in a dose-dependent manner and with high avidity. Therefore, anti-PD-L1 and anti-PD-L2 can effectively prevent the interaction of PD-1 with PD-L1 and PD-L2, respectively.

Preparation of monocyte-derived DCs

DCs were prepared from blood monocytes according to previously established protocols (23) with some modifications. PBMC were obtained from healthy donors and isolated by fractionation over Ficoll (Pharmacia, Uppsala, Sweden) gradients. To obtain monocytes, nonadherent cells were removed following a 1-h incubation at 37°C, and adherent cells were scraped from culture dishes. Cells were further enriched for monocytes by negative selection with mAb to CD19 (B4), CD20 (B1), and CD2 (T11; a gift from E. Reinherz, Dana-Farber Cancer Institute) and incubation overnight with goat anti-mouse IgG magnetic beads (Polysciences, Warrington, PA) at 4°C. Monocytes were cultured in 6-well plates at 3×10^6 cells/well (Falcon; BD Biosciences, Mountain View, CA) in X-VIVO 20 medium (BioWhittaker, Walkersville, MD) containing 1% autologous heat-inactivated serum and supplemented on days 0, 2, and 4 with 1000 U/ml IL-4 (Bio-

source International, Camarillo, CA) and 800 U/ml GM-CSF (BD Pharmingen, San Diego, CA). Maturation of iDCs was accomplished by replating the cells for 2 days at 2×10^6 cells/well in 6-well plates (Falcon; BD Biosciences) in IL-4 (1000 U/ml) and GM-CSF (800 U/ml) supplemented with IL-1 β (10 ng/ml; US Biological, Swampscott, MA), TNF- α (10 ng/ml; BD Pharmingen), IL-6 (1000 U/ml; BD Pharmingen), and PGE₂ (1 μ g/ml; Sigma-Aldrich). mDCs were consistently 90–95% CD11c⁺DR⁺ with <2% CD14⁺ cells. Where indicated, IL-10 (40 ng/ml; BD Pharmingen) was added to the maturation culture in addition to the mixture of inflammatory cytokines. The presence of a low number of CD14⁺ cells having a fibroblast-like appearance was noted after 2 days of culture with IL-10. The ability of IL-10 to convert DCs into macrophage-like cells has been previously reported (24). Before coculture with T cells, CD14⁺ macrophages were removed by incubation with anti-CD14 magnetic beads (DynaL Biotech, Great Neck, NY).

Allogenic stimulation of CD4⁺ T cells with monocyte-derived DCs

CD4⁺ T cells were isolated from peripheral blood with a CD4-positive isolation kit (DynaL Biotech) according to the manufacturer's protocol. CD4⁺ T cells were routinely >95% pure. CD4⁺ T cells (2×10^5 /well) were cocultured with immature, mature, or IL-10-pretreated mature allogenic DCs (2×10^4 /well) in triplicate in 96-well flat-bottom plates (Falcon; BD Biosciences) in 200 μ l of X-VIVO 20 (BioWhittaker) with mAb or Fab to PD-L1 (29E.2A3) and PD-L2 (24F.10C12) at various concentrations. In some experiments, CD4⁺ T cells (2×10^5 /well) were cocultured with 5×10^3 iDCs/well. Isotype-matched mAbs (IgG2b, MPC.11; IgG2a, C1.18.4) were prepared from hybridomas obtained from American Type Culture Collection (Manassas, VA) and used as negative controls. All mAbs had an endotoxin level of <2 endotoxin U/mg. Cultures were incubated for 4 days (mDC) or 5 days (iDC or IL-10-pretreated mDC) and then pulsed with 1 μ Ci/well of [³H]thymidine (New England Nuclear, Boston, MA) for 18 h. The cultures were harvested onto glass fiber filters and counted with a 1450 MicroBeta scintillation counter (PerkinElmer Wallac, Gaithersburg, MD).

Culture and FACS analysis for expression of PD-L1 and PD-L2 on T cells, monocytes, and DCs

T cells and monocytes were cultured in RPMI 1640 (Mediatech, Washington, DC) with 10% FCS and supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate (all from Life Technologies, Rockville, MD). T cells for FACS analysis were prepared from peripheral blood by negative selection. Briefly, whole blood was fractionated over Ficoll gradients, and monocytes were removed by plastic adherence. Nonadherent cells were subsequently incubated for 30 min on ice with the following mAbs: anti-CD11b (OKM1), anti-CD16 (3G8), and anti-CD20 (B1) (hybridomas from the American Type Culture Collection). T cells were then isolated by negative selection with goat anti-mouse IgG magnetic beads (Polysciences). T cells (1.5×10^5 cells/well) were activated in 96-well, round-bottom plates (Costar, Cambridge, MA) coated with 1 μ g/ml anti-CD3 (OKT3); cells were harvested each day for FACS analysis.

PBMC were isolated by adherence to plastic for 1 h and extensively washed to remove nonadherent cells. Monocytes were subsequently cultured at 5×10^6 cells/well in 6-well plates (Falcon; BD Biosciences) with 500, 167, or 56 U/ml IFN- γ (BD Pharmingen). Aliquots of cells were harvested each day for FACS analysis.

To facilitate cell surface staining of PD-L1 and PD-L2, mAbs to PD-L1 and PD-L2 were conjugated to FITC using a commercially available kit (Pierce). The staining of T cells, monocytes, and DCs followed the same basic procedure. Briefly, cells were incubated with both the optimized dilution of the FITC-conjugated anti-PD-L Abs and a corresponding lineage-specific mAb: anti-CD4-PE (T4), anti-CD8-PE (T8), anti-CD14-PE (Mo2), or anti-CD11c-PE (BU15) (all from Beckman Coulter). Cells were washed twice in cold PBS with 2% FCS and 0.02% sodium azide. Cells were fixed in 1% formaldehyde and analyzed on an EPICS XL-MCL flow cytometer (Beckman Coulter).

ELISA for cytokine analysis

Supernatants were harvested immediately before the addition of [³H]thymidine to allogenic cell cultures. IFN- γ -specific mAbs and an rIFN- γ standard (all from BD Pharmingen) were used for the detection of IFN- γ in the culture supernatants. The limit of detection was 30 pg/ml. IL-10 production was detected in culture supernatants with an ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The limit of detection was 12 pg/ml.

Histology and immunohistochemistry

Case material was obtained from Brigham and Women's Hospital in accordance with institutional policies. Immunohistochemical staining of frozen tissue sections was performed as previously described (25), using anti-PD-L1 Ab 29E.5A9 or 29E.2A3 and anti-PD-L2 Ab 24F.10C12. An AE1/AE3 cytokeratin Ab mixture (DAKO, Carpinteria, CA) was used to stain thymic epithelia. Immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections was performed as previously described (26), using microwave Ag retrieval in 10 mM citrate buffer (pH 6.0) and anti-PD-L1 Ab 29E.2A3. For both procedures, primary mouse mAb were used at optimal concentrations for detection followed by incubation with biotinylated horse anti-mouse IgG Ab (Vector Laboratories, Burlingame, CA). Detection of mAb complexes was accomplished using an avidin-biotinylated-peroxidase complex (Vector Laboratories) followed by incubation with diaminobenzidine/hydrogen peroxide. Immunohistochemical staining for the presence of PD-L1 and PD-L2 was compared with that of isotype control Abs. Comparison of tissues stained with anti-PD-L1 vs the isotype control revealed no detectable background staining on either normal or malignant tissues. Tissue sections stained with either anti-PD-L2 or the isotype control IgG2a also revealed a lack of nonspecific staining except in placenta where the IgG2a mAb demonstrated nonspecific binding to tissue macrophages (Fig. 9L).

Purification and culture of follicular DC (FDC)

FDC were isolated and cultured from tonsils (aged 2–5 years) after elective tonsillectomy as previously described (27). Adherent FDC were harvested with 5 mM EDTA in PBS and thoroughly washed. To block FcR on FDC, the cells were preincubated with 10 μ g/ml mouse Fc fragments (The Jackson Laboratory, Bar Harbor, ME) for 20 min on ice. FITC-conjugated anti-PD-L1, anti-PD-L2, or FITC-control were subsequently added, and the cells were incubated on ice for 40 min. FDC were fixed in 2% paraformaldehyde in PBS before analysis.

Results

Cell surface expression of PD-L1 and PD-L2 on monocytes, DCs, and activated T cells

mAbs to human PD-L1 and PD-L2 were selected based on their specificity, capacity to block binding to PD-1, and lack of cross-reactivity (Figs. 1 and 2). Previous reports (10, 11) have shown that IFN- γ induces PD-L1 and PD-L2 mRNA expression in PBMC. To examine PD-L1 and PD-L2 protein expression, monocytes were cultured with or without IFN- γ and incubated with anti-PD-L mAbs at the indicated time points. As shown in Fig. 3,

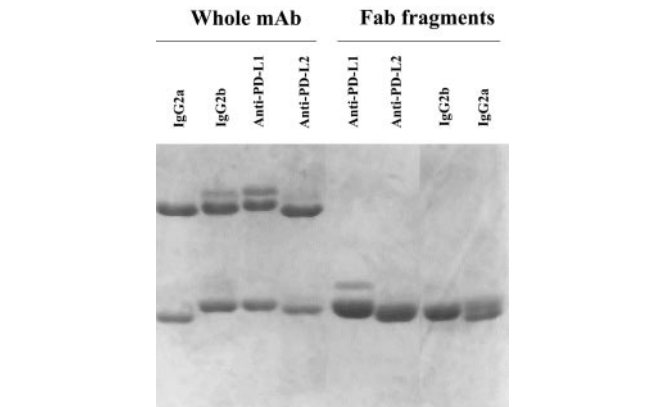


FIGURE 2. SDS-PAGE of whole mAb and Fab of anti-PD-L1 and anti-PD-L2. Whole mAb or Fab of anti-PD-L1, anti-PD-L2, or isotype controls (5 μ g/lane) were loaded on a reducing SDS-PAGE gel and subsequently stained with Coomassie blue.

ex vivo monocytes do not express either PD-L1 or PD-L2 (0 h). Culture of monocytes on tissue culture plastic in medium alone induced PD-L1 expression which peaked at 24 h and declined to background levels by 72 h. In contrast, PD-L2 was not expressed. The addition of 500 U/ml IFN- γ induced somewhat higher levels of PD-L1 expression at 24 h as compared with culture on plastic, and expression was sustained until 72 h. In addition, IFN- γ induced a low level of PD-L2 expression (Fig. 3). Lower amounts of IFN- γ induced reduced levels of PD-L1 and PD-L2 expression (data not shown).

The expression of PD-L1 and PD-L2 on different subsets of monocyte-derived DCs was examined. Monocytes were obtained from peripheral blood and cultured for 5 days in GM-CSF and IL-4 according to established protocols (23) to generate iDCs (2). To produce mDCs (2), the iDCs were incubated with a mixture of inflammatory cytokines containing IL-1 β , TNF- α , IL-6, and PGE₂ for an additional 2 days. The mDC displayed the characteristic phenotype of CD11c⁺DR⁺CD86⁺CD14⁺ with 85% of the cells

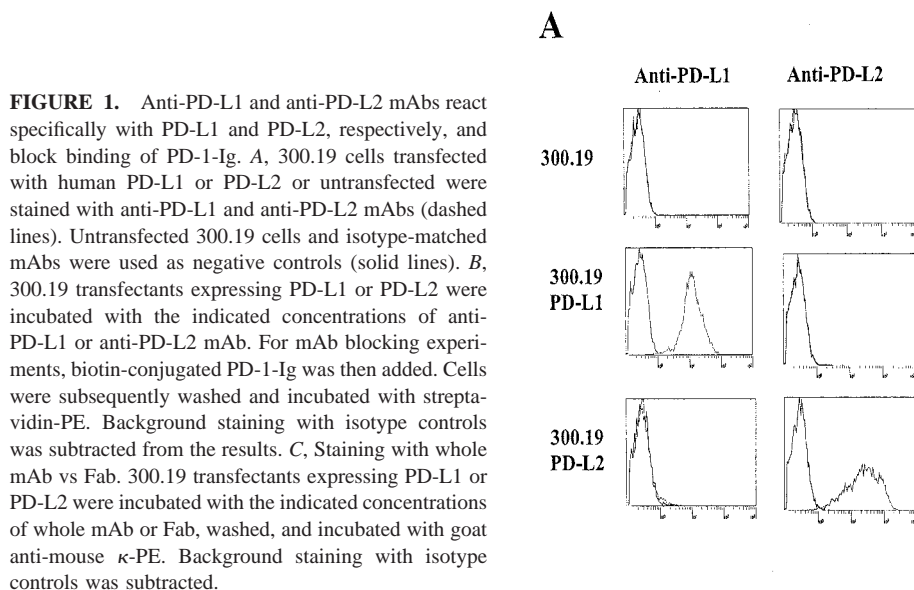
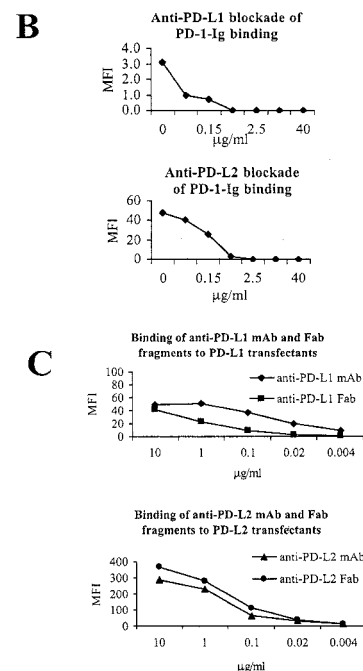


FIGURE 1. Anti-PD-L1 and anti-PD-L2 mAbs react specifically with PD-L1 and PD-L2, respectively, and block binding of PD-1-Ig. *A*, 300.19 cells transfected with human PD-L1 or PD-L2 or untransfected were stained with anti-PD-L1 and anti-PD-L2 mAbs (dashed lines). Untransfected 300.19 cells and isotype-matched mAbs were used as negative controls (solid lines). *B*, 300.19 transfectants expressing PD-L1 or PD-L2 were incubated with the indicated concentrations of anti-PD-L1 or anti-PD-L2 mAb. For mAb blocking experiments, biotin-conjugated PD-1-Ig was then added. Cells were subsequently washed and incubated with streptavidin-PE. Background staining with isotype controls was subtracted from the results. *C*, Staining with whole mAb vs Fab. 300.19 transfectants expressing PD-L1 or PD-L2 were incubated with the indicated concentrations of whole mAb or Fab, washed, and incubated with goat anti-mouse κ -PE. Background staining with isotype controls was subtracted.



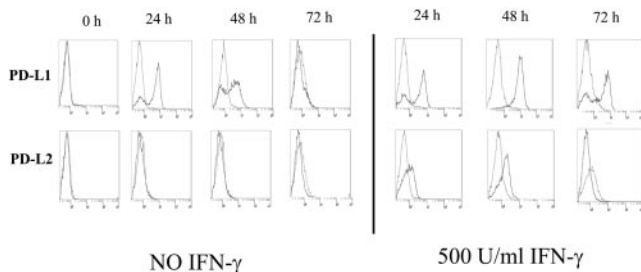


FIGURE 3. Induction of PD-L1 and PD-L2 expression on monocytes by IFN- γ . PBMC were cultured with 500 U/ml IFN- γ or medium alone. Aliquots of cells were harvested each day for 3 days and stained with anti-CD14-PE and either anti-PD-L1-FITC or anti-PD-L2-FITC mAbs (solid lines) or isotype controls (dashed lines). Zero-hour cells were analyzed directly from peripheral blood. Histograms represent staining gated on CD14 $^{+}$ cells.

staining positive for the mDC specific marker CD83 (data not shown). Furthermore, because IL-10 reduces the capacity of mDC to stimulate T cells (6, 28, 29), we reasoned that the PD-1:PD-L pathway may play a role in mediating the effects of IL-10 on mDC. Therefore, IL-10-pretreated mDC were generated by adding IL-10 to the mixture of inflammatory cytokines used to effect DC maturation. iDC, mDC, and IL-10-pretreated mDC were stained with anti-PD-L1 or anti-PD-L2 and a mAb for the lineage-specific marker CD11c. As shown in Fig. 4, iDCs uniformly express significant levels of PD-L1, suggesting that GM-CSF and/or IL-4 induces and maintains PD-L1 expression. In contrast, mDCs express higher levels of PD-L1 (mean fluorescence intensity of iDC, 4.9, and of mDC, 21.6). Treatment of mDC with IL-10 resulted in a slight decrease in PD-L1 expression levels relative to mDC; however, the percentages of PD-L1 $^{+}$ cells were comparable (Fig. 4). PD-L2 was minimally expressed on iDC. The maturation of iDC resulted in a substantial up-regulation in the level of PD-L2 expression. However, the levels of PD-L2 were reduced upon pretreatment of mDC with IL-10 (Fig. 4).

Because PD-1 can be expressed on activated T, B, and myeloid cells, we also examined PD-L expression on T cells. CD4 $^{+}$ and CD8 $^{+}$ T cells were stimulated with plate-bound anti-CD3 and stained with anti-PD-L mAbs. Unstimulated T cells did not express PD-L1 or PD-L2. CD4 $^{+}$ and CD8 $^{+}$ T cells both demonstrated a progressive increase in the intensity of PD-L1 expression as well

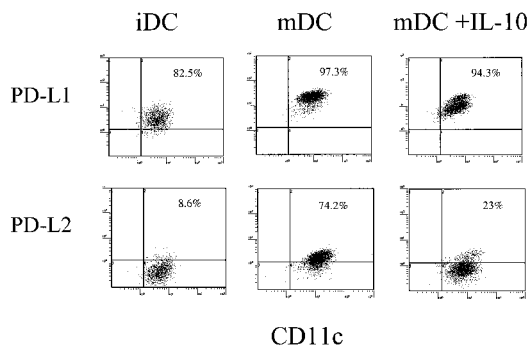


FIGURE 4. PD-L1 and PD-L2 expression on iDC, mDC, and IL-10-pretreated mDC. DC were prepared from PBMC as described in *Materials and Methods*. iDC were harvested on day 5 of culture. iDC were incubated for an additional 2 days with inflammatory cytokines to produce mDC. IL-10-pretreated mDC were cultured similarly to mDC with the addition of IL-10 (40 ng/ml) during the final 2 days of culture. DCs were stained with anti-CD11c-PE and either anti-PD-L1-FITC or anti-PD-L2-FITC and analyzed by flow cytometry.

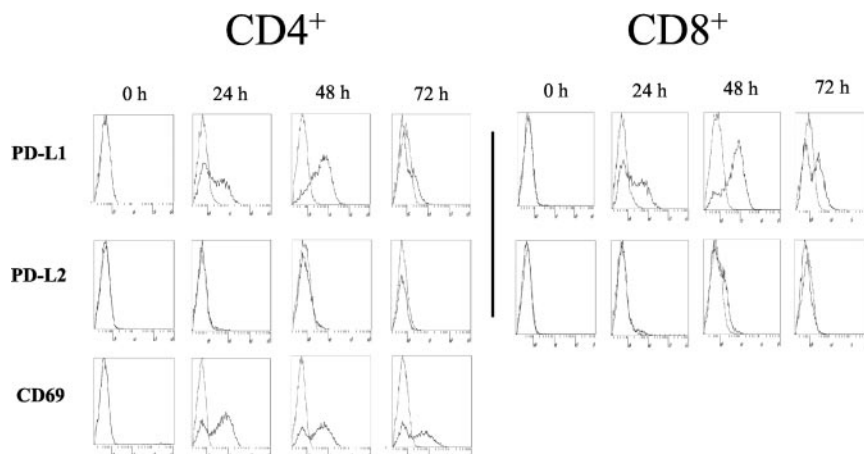
as the percentage of positive cells through 48 h (Fig. 5). The level of PD-L1 expression diminished at 72 h in both CD4 $^{+}$ and CD8 $^{+}$ T cells (Fig. 5). Peak expression of PD-L1 on CD4 $^{+}$ T cells occurred 24 h later than peak expression of the activation marker, CD69. PD-L2 expression was not induced on CD4 $^{+}$ T cells upon activation, but very low levels of PD-L2 expression were seen at 48 h on CD8 $^{+}$ T cells and diminished to background levels at 72 h (Fig. 5). Stimulation of CD4 $^{+}$ and CD8 $^{+}$ T cells with plate-bound anti-CD3 plus anti-CD28 modestly increased PD-L1 expression but did not induce PD-L2 expression (data not shown).

Blockade of the PD-L:PD-1 pathway enhances T cell activation in an MLR

Previous investigations using PD-L transfected fibroblasts to present OVA peptide to D011.10 TCR transgenic CD4 $^{+}$ T cells revealed that the PD-L:PD-1 pathway exerts its greatest inhibitory effect at relatively low concentrations of Ag (11). Therefore, we compared the effect of PD-L blockade on CD4 $^{+}$ T cell alloresponses using DC of weak (IL-10-pretreated mDC or iDC) (2, 3, 5–7, 29) vs strong (mDC) stimulatory capacity. Increasing concentrations of anti-PD-L Fab were added to cultures of CD4 $^{+}$ T cells and allogenic DCs. iDC stimulated a weak alloresponse in the presence of isotype control Fab (Fig. 6A). In contrast, the addition of 20 μ g/ml anti-PD-L2 Fab resulted in a 3-fold increase in T cell proliferation compared with cultures that received isotype control Fab (Fig. 6A). iDC cultures treated with anti-PD-L1 Fab showed no increase in T cell proliferation above the isotype control. However, the addition of 20 μ g/ml anti-PD-L1 and anti-PD-L2 Fab together demonstrated a 4-fold increase in T cell proliferation, slightly greater than anti-PD-L2 alone. IL-10-pretreated mDC stimulated a very weak alloresponse in the presence of isotype control Fab. In contrast, the addition of anti-PD-L2 Fab at 20 μ g/ml resulted in a 9-fold increase in T cell proliferation compared with that of the isotype control (Fig. 6B). The addition of anti-PD-L1 Fab to IL-10-pretreated mDC resulted in only a slight increase in T cell proliferation. However, the combination of anti-PD-L1/anti-PD-L2 Fabs (20 μ g/ml) resulted in an 11-fold increase in T cell proliferation compared with that of the isotype control. As expected, mDC stimulated a robust alloresponse. Anti-PD-L2 Fab or the combination of anti-PD-L1 and anti-PD-L2 Fabs enhanced T cell proliferation to a similar extent (1.4-fold increase over isotype controls), but the effect was less dramatic than with iDC or IL-10-pretreated mDC (Fig. 6C). The treatment of mDC with anti-PD-L1 Fab resulted in a modest increase in T cell proliferation compared with that of cultures treated with an isotype control.

The blockade of PD-Ls appeared to have the greatest effect on T cell stimulation under conditions of relatively weak TCR stimulation, i.e., iDC and mDC treated with IL-10. To further examine the effect of PD-L blockade under conditions of a weak T cell stimulus, a comparison between two different ratios of T cells and iDC was performed. Cocultures of allogenic CD4 $^{+}$ T cells and iDC at 10:1 and 10:0.25 were treated with 20 μ g/ml anti-PD-L1, anti-PD-L2, or both anti-PD-L Fab, or the appropriate isotype controls (Fig. 6, D and E). Addition of anti-PD-L1 Fab either modestly enhanced (Fig. 6D) or did not affect (Fig. 6E) the proliferation of the CD4 $^{+}$ T cells. In contrast, the addition of anti-PD-L2 Fab to the cultures substantially increased T cell proliferation, while the combination of both anti-PD-L Fabs provided an additive stimulation of proliferation. Anti-PD-L2 was more effective at increasing T cell proliferation at the lower iDC concentration than at the higher concentration (10.6- vs 5-fold) while the combination of both anti-PD-L Fabs also resulted in a similar pattern of enhanced proliferation (9.3-fold at 10:1 and 11.7-fold at 10:0.25).

FIGURE 5. PD-L expression on anti-CD3-stimulated T cells. Purified CD4⁺ or CD8⁺ T cells were incubated in 96-well plates coated with 1 μ g/ml anti-CD3, and aliquots of cells were harvested each day for 3 days and stained with anti-PD-L1-FITC, anti-PD-L2-FITC, or anti-CD69-PE (CD4⁺ only) (solid lines) or isotype controls (dashed lines) for subsequent analysis by FACS. Histograms represent staining gated on CD4⁺ or CD8⁺ cells.



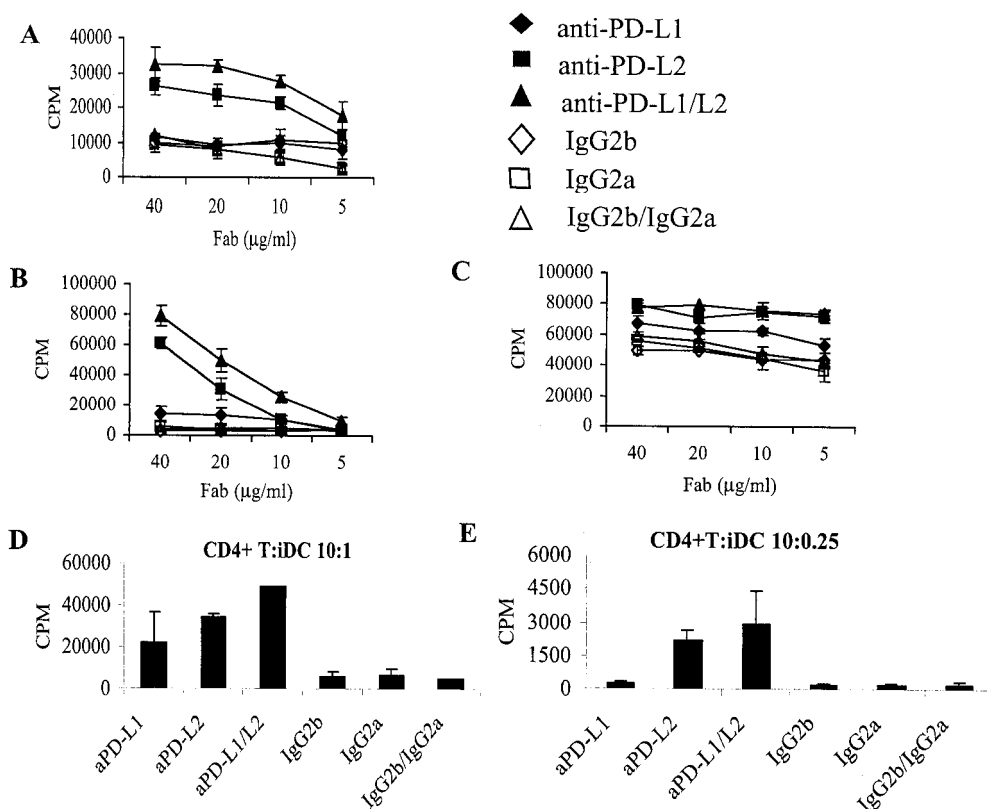
The effect of PD-L blockade on IFN- γ and IL-10 production by allogenic CD4⁺ T cells stimulated with iDC, mDC, or IL-10-pretreated mDC was examined (Fig. 7). The combination of anti-PD-L1 and anti-PD-L2 Fabs stimulated IFN- γ and IL-10 production in all DC/T cultures. The enhancement was generally greatest with the weaker DC populations (iDC and IL-10-pretreated mDC) than with the mDC. However, mDC/T cultures treated with anti-PD-L1 and anti-PD-L2 Fab showed a pronounced increase in the production of IL-10 compared with Fab isotype control-treated cultures. Furthermore, either anti-PD-L1 Fab or anti-PD-L2 Fab alone increased IL-10 production from mDC/T cultures (Fig. 7B). While anti-PD-L2 Fab enhanced proliferation more than anti-PD-L1 Fab, each Fab equally stimulated IFN- γ and IL-10 production from iDC/T and mDC/T cultures. With iDC/T cultures, little IFN- γ was produced in the presence of control Fab; however, the stimulation by PD-L blockade was striking (Fig. 7D). With IL-10-pretreated mDC/T cultures, anti-PD-L1 Fab had little effect on

IFN- γ production, but anti-PD-L2 Fab and the combination of Fabs strongly stimulated IFN- γ production (Fig. 7F). IL-10-pretreated mDC/T control cultures produced no detectable IL-10, and a small increase above background levels was seen with the combination of anti-PD-L1 and anti-PD-L2 Fabs (Fig. 7C).

PD-L expression on normal tissues and tumors

In contrast to the B7 family members B7-1 and B7-2, mRNA for PD-L1 and PD-L2 are expressed in both lymphoid and nonlymphoid tissues (10). PD-L1 and PD-L2 mRNAs are both highly expressed in placenta, heart, lymph node, and thymus (11), but the cell types expressing these proteins are not known. Figs. 8 and 9 demonstrate the staining of these tissues with anti-PD-L1, anti-PD-L2, or isotype-matched control mAb. In fetal thymus, both cortical and medullary tissues demonstrated PD-L1 expression (Figs. 8A and 9A). This pattern of expression coincides with the staining of thymic tissue with anti-cytokeratin mAb specific for

FIGURE 6. Anti-PD-L1 and anti-PD-L2 Fabs increase CD4⁺ T cell proliferation in allogenic cultures with iDC, IL-10-pretreated mDC, and mDC. Allogenic CD4⁺ T cells (2×10^5 /well) were cocultured with 2×10^4 DC: iDC (A), IL-10-pretreated mDC (B), and mDC (C). A–C are representative of three experiments. Comparable results were seen in seven similar experiments with IL-10-pretreated mDC upon blockade of PD-L1 and/or PD-L2 with whole mAb (data not shown). D and E, Allogenic CD4⁺ T cells (2×10^5 /well) were cocultured with 2×10^4 iDC (D) or 5×10^3 iDC (E). The SD are indicated.



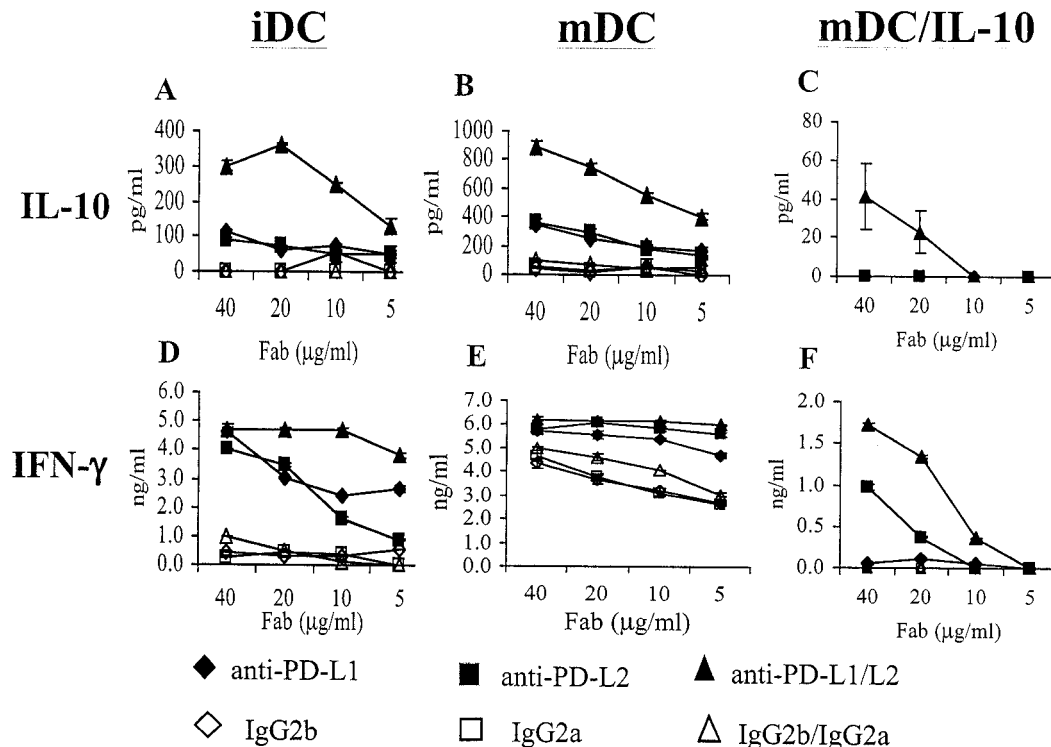


FIGURE 7. IFN- γ and IL-10 are elevated in allogenic cultures of CD4⁺ T cells and various DC populations treated with Fab to PD-L1 and PD-L2. Allogenic iDC (A and D), mDC (B and E), or IL-10-pretreated mDC (C and F) (2×10^4 cells/well) were cocultured with 2×10^5 CD4⁺ T cells/well in 96-well plates. Cultures containing iDC or IL-10-pretreated mDC were incubated for 5 days, and cultures with mDC were incubated for 4 days before supernatants were removed for cytokine analysis. The levels of IL-10 and IFN- γ production were determined in duplicate by ELISA and the SD are indicated.

epithelial cell markers suggesting that PD-L1 is expressed by thymic epithelial cells (Fig. 8C). In contrast, the expression of PD-L2 is more confined to the medullary areas of the thymus (Figs. 8B and 9C). The expression of the macrophage/DC marker CD68 demonstrates a similar tissue distribution suggesting that PD-L2 is expressed by both medullary macrophages/DC and epithelial cells (Fig. 8D). In germinal centers of tonsil, PD-L1 and PD-L2 were expressed in a pattern consistent with the distribution of FDC and macrophages (Fig. 8, E and F). Consistent with this staining pattern, we have found moderate expression of PD-L1 and PD-L2 on cultures of primary FDC-like cells (Fig. 10). Human placenta shows strong expression of PD-L1 on syncytiotrophoblasts (Fig. 8G and 9I), the cells found at the maternal-fetal interface, but expression was lower on the underlying cytotrophoblasts that give rise to syncytiotrophoblasts. PD-L2 is not expressed on trophoblasts but is highly expressed on the endothelium of placental blood vessels (Figs. 8H and 9K). The isotype control for the anti-PD-L2 mAb, IgG2a, demonstrated nonspecific staining on tissue macrophages of the placenta known as Hofbauer cells (Fig. 9L); consequently, we believe that the staining of Hofbauer cells by PD-L2 is nonspecific. Fetal cardiac endothelium strongly expressed PD-L1, and myocardium expressed low levels (Fig. 8I). In contrast, adult cardiac tissue had strong expression of PD-L1 in the myocardium and lower expression on endothelial cells (data not shown). In fetal heart, PD-L2 was expressed at low levels in myocardium and endothelial cells (Fig. 8J).

PD-L1 is expressed on the cell surface of a number of human breast cancer cell lines, and PD-L1 mRNA is expressed on a number of murine tumor cell lines (11). PD-L1 expression in situ was examined in a variety of human tumors (Figs. 8 and 9; Table I). In

hematologic malignancies, PD-L1 was not expressed on any of 16 B cell non-Hodgkins lymphomas examined; however, PD-L1 was expressed on some primary T cell lymphomas, particularly anaplastic large-cell lymphomas (Fig. 8K and Table I). PD-L1 was expressed on most thymic epithelial tumors including benign (67%) and invasive (100%) thymomas as well as thymic carcinoma (88%) (Table I). Most carcinomas of other sites showed staining with anti-PD-L1 mAb (Table I). Fig. 8 shows staining of squamous cell carcinoma of the tongue (Fig. 8L) and adenocarcinoma of the colon (Figs. 8M and 9S). Most breast carcinomas demonstrated staining with anti-PD-L1, while all carcinomas of the cervix, colon, larynx, and lung examined expressed PD-L1 (Table I). In sections of breast containing cells with both normal and malignant histology, PD-L1 was highly expressed on malignant invasive ductal carcinoma cells but poorly expressed on adjacent cells still exhibiting normal structure (Figs. 8, N and O, and 9Q).

Discussion

To better understand the function of PD-Ls, we have developed anti-PD-L1 and PD-L2 mAbs which block engagement with PD-1. Using these mAbs, we find that monocytes do not constitutively express either PD-L1 or PD-L2 but expression can be induced by IFN- γ , consistent with our previous data on mRNA expression (10, 11). iDC derived by culture of monocytes in GM-CSF and IL-4 express abundant PD-L1 and low levels of PD-L2. Maturation of DC with a mixture of inflammatory cytokines up-regulates both PD-L1 and PD-L2 expression. Maturation in the presence of IL-10 leads to reduced levels of both PD-L1 and PD-L2. Unstimulated T cells do not express PD-Ls, but PD-L1 is induced on both CD4⁺ and CD8⁺ T cells following activation. PD-L2 is not expressed on

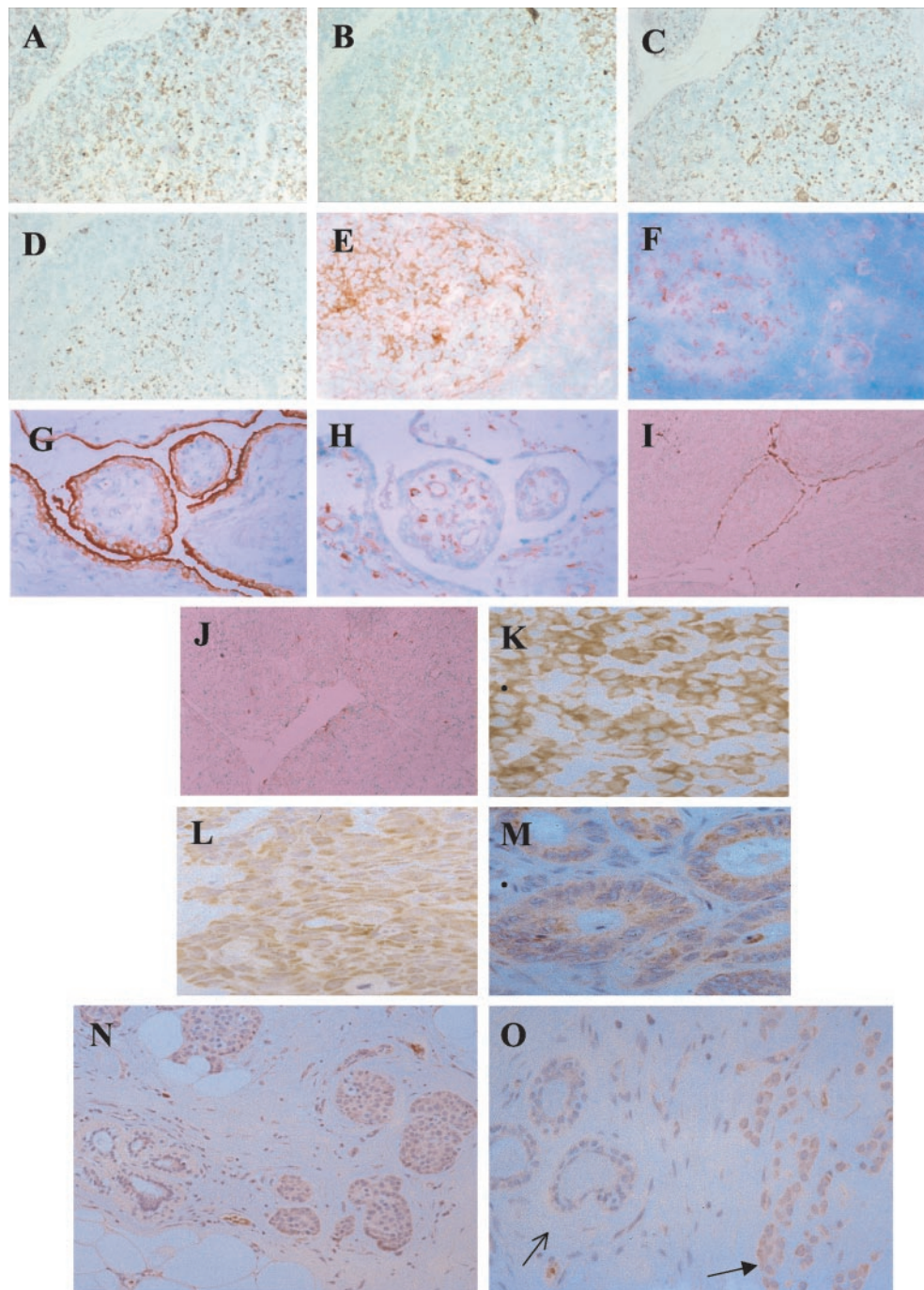


FIGURE 8. PD-L1 and PD-L2 demonstrate distinct patterns of expression in thymus, germinal center, placenta, cardiac tissues, and tumors. All tissues were processed as paraffin sections as described in *Materials and Methods* (except *A–D*, *F*, *H*, and *J*, which were frozen sections) and subsequently counterstained with methyl green. Shown are fetal thymus, anti-PD-L1 (*A*), anti-PD-L2 (*B*), anti-cytokeratin mAb mixture AE1/AE3 (*C*), and anti-CD68 (*D*); tonsillar germinal center, anti-PD-L1 (*E*) and anti-PD-L2 (*F*); placenta, anti-PD-L1 (*G*) and anti-PD-L2 (*H*); and fetal cardiac tissue, anti-PD-L1 (*I*) and anti-PD-L2 (*J*). *A–D*, *F–H*, and *J*, Magnified $\times 100$. *E* and *I*, Magnified $\times 200$. *K*, Anaplastic large cell lymphoma, anti-PD-L1, magnified $\times 400$. *L*, Squamous cell carcinoma of the tongue, anti-PD-L1, magnified $\times 200$. *M*, Adenocarcinoma of the colon, anti-PD-L1, magnified $\times 200$. *N* and *O*, Invasive ductal carcinoma of the breast, anti-PD-L1, magnified $\times 100$. Open-ended arrows (\rightarrow) in *O* indicate tissue with normal structure and close-ended arrows (\rightarrow) indicate malignant tissue. Staining with isotype controls was negative except for IgG2a staining of macrophages in placenta (Fig. 9).

CD4⁺ T cells following stimulation with anti-CD3, although low levels may be expressed by CD8⁺ T cells.

We examined the functions of PD-L1 and PD-L2 by analyzing the consequences of PD-L blockade during T cell responses initiated by allogeneic DC. Blockade of PD-Ls increased T cell proliferation and cytokine production. Both Fab and whole mAb to PD-L1 and PD-L2 enhanced T cell proliferation and cytokine production, and only the results with Fab are shown. The use of monomeric Fab eliminates the possibility that the results are due to FcR-mediated effects or signal transduction by cross-linking of PD-L. No contaminating whole mAb was detectable in the Fab preparations, and the binding characteristics of the Fab were comparable to that of the whole mAb (Figs. 1 and 2).

We compared the effects of PD-L blockade upon DCs of different potency. mDC are powerful APC, whereas iDC or IL-10-

pretreated mDC are much less potent. The enhancement of T cell activation by PD-L blockade was stronger with IL-10-pretreated mDC or iDC than with mDC. The combination of anti-PD-L1 and anti-PD-L2 Fab consistently gave the greatest increase in T cell proliferation and cytokine production. Surprisingly, given the higher expression of PD-L1 on DC, PD-L2 blockade accounted for most of the enhancement of T cell proliferation, suggesting that human PD-L2 is more potent. Generally, PD-L1 or PD-L2 blockade enhanced cytokine production equivalently. This is analogous to results with blockade of B7-1 and B7-2, where blockade of B7-2 has greater effects. The interactions of B7-1 and B7-2 with their ligands, CD28 and CTLA4, were first thought to have an affinity difference of ~ 20 -fold. More recent studies have shown that the weakest interaction (B7-2/CD28) has a 10,000-fold lower avidity than the highest interaction (B7-1/CTLA4) due to dimerization and

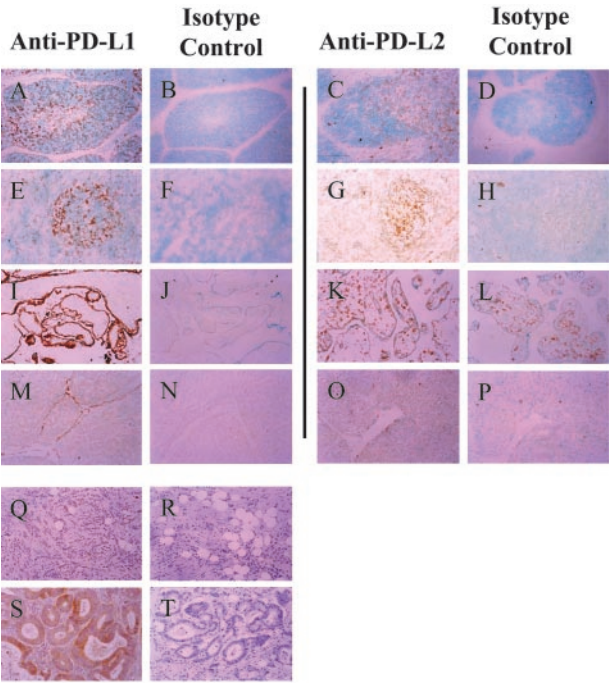


FIGURE 9. Immunohistochemistry of normal and malignant tissue sections stained with anti-PD-L1, anti-PD-L2, and isotype controls. Tissue sections stained with anti-PD-L1 or IgG2b isotype-matched control, respectively, are the following: A and B, fetal thymus; E and F, tonsil; I and J, placenta; M and N, fetal heart; Q and R, infiltrating lobular carcinoma of breast; S and T, colonic adenocarcinoma. Tissue sections stained with anti-PD-L2 or IgG2a isotype control, respectively, are the following: C and D, fetal thymus; G and H, tonsil; K and L, placenta; and O and P, fetal heart.

small monomeric affinity differences (30). Our studies suggest that PD-L2 is the more important PD-L on monocyte-derived DC and further studies will try to establish a structural basis for this.

With iDC as APC, PD-L1 or PD-L2 blockade strongly augmented IFN- γ production and modestly enhanced IL-10 production. IL-10-pretreated DC stimulated only very low levels of proliferation and cytokine production in the presence of control Fab but high levels of proliferation and moderate levels of IFN- γ and IL-10 in the presence of PD-L1 and PD-L2 Fab. With mDC, PD-L1 or PD-L2 blockade modestly augmented the already high level of IFN- γ production and strongly enhanced IL-10 production. Furthermore, with reduced numbers of iDC, the relative increase in T cell proliferation was enhanced compared with cultures

Table I. PD-L1 expression in tumors

Tumor	No. PD-L1 Positive/No. Observed
Thymic neoplasms	
Benign thymoma	10/15
Invasive thymoma	11/11
Thymic carcinoma	7/8
Lymphoid neoplasms	
PTCL	7/11
B cell non-Hodgkin lymphoma	0/16
Carcinomas	
Bladder, transitional cell	4/6
Breast	
Invasive ductal	5/6
Invasive lobular	4/6
Cervix, squamous	3/3
Colon, adenocarcinoma	6/6
Endometrium	1/3
Gallbladder	1/1
Larynx, squamous	3/3
Liver	4/4
Lung	
Squamous	8/8
Adenocarcinoma	6/6
Ovary	1/3
Salivary	5/6
Stomach, adenocarcinoma	2/3
Thyroid, follicular	4/5

with higher numbers of iDC. Thus, blockade of the PD-L:PD-1 pathway had the greatest impact on T cell activation under conditions of relatively weak stimulation. These results are consistent with the results of Latchman et al. (11) who showed that stimulation of D011.10 TCR transgenic T cells with CHO-I-A^d/B7-2 transfectants that expressed either PD-L1 or PD-L2 demonstrated a striking reduction in T cell proliferation, particularly at lower concentrations of peptide Ag, with concomitant reductions in IL-2, IL-4, and IFN- γ production.

Recent data have revealed that Ag presentation by iDCs has a role in establishing and maintaining T cell tolerance (2, 5). IL-10-pretreated mDCs have been reported to anergize allogenic CD4⁺ T cells (3, 6, 7). iDCs and IL-10-pretreated mDCs express lower levels of B7-1, B7-2, and class II MHC molecules than mDC. This lower level of stimulatory molecules has been proposed to account for their poor capacity to stimulate T cells (2, 6, 29); however, expression of comparably low amounts of MHC and B7 on transfectants is sufficient for efficient T cell activation (Ref. 31 and G. Freeman, unpublished data). An IL-10-pretreated mDC with its PD-Ls blocked stimulated T cell proliferation in a manner broadly comparable to an mDC (Figs. 6 and 7). Our interpretation is that T cells are responding to and “summing up” both positive (CD28 ligands) and negative (PD-1 and CTLA4 ligands) signals from the DC. We believe these data are consistent with the hypothesis that iDC have a balance of stimulatory vs inhibitory molecules that favors inhibition and may explain why iDC are poor stimulators. This may happen at the level of phosphorylation of TCR proximal kinases because PD-1 has been shown to activate Src homology 2-domain-containing tyrosine phosphatase 2 (11). Although the expression of PD-Ls is somewhat lower on iDC or IL-10-treated mDC, the expression of B7-1 and B7-2 has been shown to be much lower than on mDC. In the mDC, the high levels of B7-1, B7-2, and MHC provide a sufficiently strong stimulatory signal to overwhelm the negative signals delivered via the PD-L:PD-1 pathway, resulting in T cell activation. Because the effect of PD-L blockade is so potent on iDC and IL-10-treated mDC, we believe that PD-Ls make an important contribution to the poor stimulatory capacity of

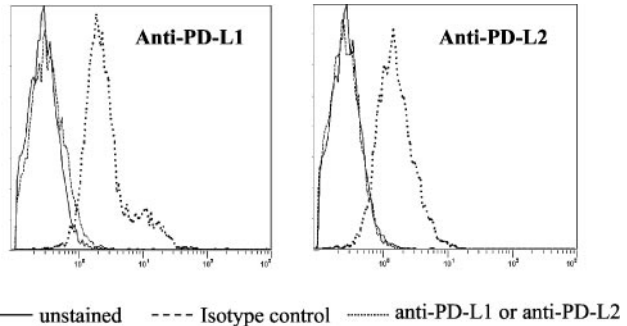


FIGURE 10. Tonsil-derived FDC-like cells express PD-L1 and PD-L2. FDC-like cells were derived from tonsils and cultured as described in *Materials and Methods*. The cells were subsequently stained with anti-PD-L1-FITC, anti-PD-L2-FITC, or control mAbs, or unstained and analyzed by FACS.

iDC. Given its immunoinhibitory function, the PD-L:PD-1 pathway may play a role in enhancing the development of anergic or regulatory T cells by limiting T cell activation.

CTLA-4 and PD-1 are both immunoinhibitory but most potently act on T cell populations of different avidities. CTLA-4 is most strongly induced and transported to the immunological synapse by a strong TCR signal (32). For this reason, CTLA-4 most strongly inhibits T cells that express a TCR with high avidity (33). CTLA-4 engagement limits the expansion of the primed pool of CD4⁺ T cells and, by preferentially inhibiting T cells with the highest avidity TCR, increases the diversity of the TCR repertoire (34). In contrast, PD-1 is more strongly induced in T cells that have received a weak TCR signal and most potently inhibits T cells that have received a weak TCR signal (11). PD-1 engagement would be predicted to preferentially inhibit low-avidity Ag receptors and thus select for higher avidity TCR or B cell receptors, leading to the preferential expansion of immunodominant clones.

Our data demonstrate that IFN- γ is a major inducer of PD-L expression. IFN- γ ^{-/-} mice have been shown to have a defect in the induction of PD-L1 (35). One of the major surprises in IFN- γ ^{-/-} mice is that many immune responses, such as allograft rejection, experimental allergic encephalomyelitis, and collagen-induced arthritis, are much more severe. This suggests that immunoinhibition is an essential function of IFN- γ and is consistent with IFN- γ -mediated induction of the immunoinhibitory PD-Ls. Badovinac et al. (36) have shown that one of the consequences of IFN- γ deficiency is the persistence of T cells recognizing minor epitopes in the context of infection with lymphocytic choriomeningitis virus, i.e., lack of selection of immunodominant TCR epitopes, perhaps as a consequence of reduced PD-L expression.

In contrast to our results, other studies have reported that PD-L1 and PD-L2 function as stimulators of T cell responses. Dong et al. (21) and Tamura et al. (37) have shown that PD-L1-Ig (B7-H1-Ig) moderately costimulates T cell proliferation and enhances the secretion of IL-10. Tseng et al. (22) have reported that PD-L2-Ig (B7-DC-Ig) strongly costimulates T cell proliferation and enhances the secretion of IFN- γ . These results are very similar to the ones we have obtained with blocking mAbs and caused us to consider whether the Ig fusion proteins used in these studies might be functioning as antagonists rather than as agonists. Support for this idea comes from an independent group (S. Yoshinaga and S. Suggs, personal communication). Yoshinaga and Suggs made a PD-L1-Ig fusion protein and found that, when bound to tissue culture plastic with anti-CD3, it inhibited T cell activation. However, when the same PD-L1-Ig and anti-CD3 were bound to high-binding capacity ELISA plates, T cell proliferation was stimulated. One explanation for these results is that, at high density, PD-L1-Ig is binding PD-1 in a monomeric fashion and competitively blocking PD-1 signal transduction. As a consequence, the PD-L-Ig would demonstrate the stimulatory results we have described in this study with blocking mAbs. Differences in the hinge domain of the Ig fusion protein or its dimerization might also contribute to differences in cross-linking of PD-1. Another way to reconcile these contradictory results might be that, similar to the B7:CD28/CTLA4 pathway, a second receptor for PD-L might exist with the capacity to deliver a stimulatory signal like CD28. Arguing against this possibility is the fact that when PD-1^{-/-} T cells are stimulated with PD-L1-Ig and anti-CD3 mAb, no increase in proliferation over that of anti-CD3 alone is observed (10). In addition, PD-L1-Ig does not detectably bind to unstimulated T cells (G. Freeman, unpublished data).

The staining of tissue sections with anti-PD-L1 and anti-PD-L2 illustrates that PD-Ls have a wider tissue distribution than other B7 family members. Although RNA blot analysis suggested a similar

range of expression of PD-L1 and PD-L2, immunohistochemistry reveals distinct differences in expression within tissues. In the placenta, PD-L1 shows very strong expression on syncytiotrophoblasts, the cells that comprise the fetal-maternal interface. In contrast, PD-L2 is not expressed on syncytiotrophoblasts but is strongly expressed on endothelial cells. PD-L1 and PD-L2 may play an immunosuppressive role in the placenta by down-regulating allogenic T cell responses.

In the thymus, PD-L1 is primarily expressed on cortical and medullary thymic epithelial cells while PD-L2 shows stronger staining on medullary epithelial cells. PD-1 is transiently expressed at low levels on CD4⁻CD8⁻ (double-negative (DN)) T cells late in the transition phase from DN to double-positive (DP) and expression is lost after the transition to DP (38). PD-1 deficiency alters central tolerance by affecting selection in the thymus after TCR β rearrangement in DN T cells, resulting in less strict selection and an increase in DP T cells. Despite more DP T cells, PD-1 deficiency also affected positive selection resulting in a reduced number of single-positive CD8 T cells (38). Thus the PD-L:PD-1 pathway has a role in thymic selection, probably by setting thresholds for thymocyte activation. Mutations in the winged-helix transcription factor, Whn, which is defective in the nude mouse, result in the loss of expression of PD-L1 mRNA in thymic epithelial cells (39).

The expression of PD-L1 and PD-L2 on FDCs in the germinal center may have important implications for B cell activation. PD-1-deficient mice on the C57BL/6 background develop a lupus-like arthritis and glomerulonephritis, suggesting a dysregulation of B cell activation (20). PD-L expression on FDCs may attenuate B cell activation signals in the germinal center, thereby setting thresholds for B cell activation and regulating affinity maturation.

Fetal heart had strong expression of PD-L1 on endothelium and weak expression on cardiomyocytes. In adult heart, PD-L1 expression was higher on cardiomyocytes and lower on endothelium. PD-L2 was expressed at low levels on cardiomyocytes and endothelial cells in fetal cardiac tissue. Interestingly, PD-1-deficient mice on the BALB/c background develop a fatal dilated cardiomyopathy (40) characterized by anti-cardiomyocyte Abs, suggesting that PD-Ls may play a role in attenuating lymphocyte activation within cardiac tissues.

PD-L1 was not expressed on B cell hematologic malignancies but was strongly expressed on anaplastic large cell lymphoma, a T cell malignancy. This parallels the normal expression in B and T cells, where expression is seen on activated T cells but little is observed on activated human B cells or B cell lines (Fig. 5 and data not shown).

PD-L1 was expressed on a broad spectrum of carcinomas, tumors of epithelial origin. In sections of breast that contained both normal and malignant cells, expression of PD-L1 was much higher on the malignant cells. CD8⁺ T cell responses are particularly important in antitumor immunity. Carter et al. (12) have shown that PD-Ls inhibit CD8 T cell activation more effectively than CD4 T cell activation, and Iwai et al. (41) have shown that PD-L1 inhibits CD8 T cell-mediated cytotoxicity. Tumors may exploit the immunosuppressive properties of PD-L1 on CD8 T cell activation and cytotoxicity to attenuate the antitumor immune response. Because most identified tumor Ags are self-Ags to which high affinity TCR have been deleted in the thymus, the capacity of PD-Ls to inhibit responses to weak Ags may be particularly relevant. Given the enhancement of T cell responses upon blockade of PD-Ls, and the stimulatory effect of blockade of other inhibitory molecules such as CTLA-4, anti-PD-L mAbs may be useful therapeutic tools for improving antitumor T cell responses.

Because anti-PD-L mAbs enhance the capacity of DCs to stimulate T cell activation and cytokine production, these reagents may

be useful in improving the stimulatory capacity of DC in vaccination protocols. Anti-PD-L mAbs may have therapeutic potential in the clinical setting where techniques for stimulating T cells with DCs are currently being investigated.

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